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Anti-GBM glomerulonephritis in mice lacking nitric oxide synthase type 2
Rapid Communication

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Anti-GBM glomerulonephritis in mice lacking nitric oxide synthase type 2. ***Rapid Communication***. Nitric oxide is synthesized in experimental immune complex glomerulonephritis due to local induction of type 2 nitric oxide synthase (NOS2). To determine the role of NOS2, the course of accelerated anti-glomerular basement membrane glomerulonephritis (anti-GBM) was examined in mice homozygous for disruption of the NOS2 gene compared with heterozygous littermates. Disease in the wild type strain (129Sv) was characterized by heavy albuminuria, glomerular neutrophil and macrophage infiltration and glomerular thrombosis. NOS2, interleukin 1B (IL-1β) and tumor necrosis factor α (TNFα) mRNA were induced by 24 hours. The NOS2-deficient mutant mice and the heterozygous mice displayed early (24 hr) and full autologous phase (day 6) injury indistinguishable from the wild-type mice. The equivalent degree of albuminuria and glomerular inflammation indicates that NOS2 does not play an essential role in this form of glomerulonephritis in the mouse.

In experimental immune complex glomerulonephritis nitric oxide (NO) is synthesized by nephritic glomeruli [1–4]. NO synthesis results from the induction of the high output, calcium independent isoform of nitric oxide synthase (iNOS or NOS2); NOS2 mRNA can be demonstrated in glomeruli by ribonuclease protection assay [5] and reverse transcriptase (RT)-polymerase chain reaction (PCR) [6, 7] and NOS2 protein by immunohistochemistry [8] in models of glomerulonephritis. The role of NOS2 in glomerulonephritis, however, remains controversial. NO has the potential to increase glomerular injury by its direct cytotoxic actions [9], but may also have protective actions by improving glomerular hemodynamics [10], inhibiting thrombosis [11] and adhesion molecule expression [12], and scavenging other free radicals such as superoxide [13]. Experiments to define a role for NOS2 have been hampered by the lack of completely selective inhibitors of NOS2; the major NOS inhibitors also inhibit endothelial NOS3 leading to hypertension, a factor known to exacerbate glomerulonephritis [14]. Some groups have therefore used indirect methods to manipulate NOS2 activity, such as alteration of substrate availability either by dietary supplementation with L-arginine [15] or administration of arginase [16] to deplete circulating arginine. Overall the results are inconclusive with some experiments pointing to a toxic role for NO [17, 18] and others suggesting that it is protective [16]. Therefore, in order to determine the role of NOS2 in glomerulonephritis, we have now examined the course of glomerulonephritis in mice that are homozygous for a disruption of the NOS2 gene and compared them with heterozygous littermates with functional NOS2.

Key words: glomerulonephritis, nitric oxide synthase, knock-out mice, albuminuria.

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METHODS

Mice

The derivation of mice with genetically disrupted NOS2 gene has been previously described [19]. The chimeras were bred into the inbred 129 strain (Harlan Olac, Bicester, UK). The mice were kept in an isolator at the University of Glasgow and maintained as homozygous or heterozygous littermates. DNA from each individual mouse was examined by Southern blot analysis to confirm the genotype for NOS2. Adult male mice (8 to 10 weeks of age were transferred to Imperial College School of Medicine where experiments were performed. Mice were negative for common murine viral pathogens by sera analyses.

Preliminary experiments establishing the model of accelerated anti-glomerular basement membrane (anti-GBM) glomerulonephritis in the 129Sv mouse and techniques for mouse glomerular isolation were performed on adult male 129Sv mice from Harlan Olac.

Induction and monitoring of glomerulonephritis

Mice were immunised with a single intraperitoneal dose of 0.2 mg rabbit IgG (Sigma, Poole, UK) in Freund's complete adjuvant (Sigma). Rabbit anti-mouse glomerular basement membrane antibody (globulin concentration 15 mg/ml) [20] was given intravenously seven days later. In preliminary experiments doses of between 0.2 to 3 mg anti-GBM globulin per mouse were used to establish a reproducible model of accelerated anti-GBM nephritis

with glomerular NOS2 induction in the 129Sv mouse strain. Mice were killed at one, three or six days.

In the two principal experiments described here, NOS2 deficient and NOS2 heterozygous mice received either 0.6 mg anti-GBM globulin and were killed at 24 hours, or 0.3 mg anti-GBM globulin and were killed 24 hours or six days later. Kidneys were removed for glomerular isolation, histology and immunohistochemistry. Mice were housed in metabolic cages for urine collection.

Urinalysis

Urine was collected for 24 hour periods. Albuminuria was measured by rocket immunoelectrophoresis using sheep anti-rat albumin antibody [21] (Biogenesis Ltd., Poole, UK). Creatinine was measured by the Jaffe reaction on an automatic analyzer.

Glomerular isolation

Magnetizable iron oxide was prepared as described [22]. A diethyl pyrocarbonate (DEPC) treated sterile suspension of iron oxide (1.25% wt/vol) in 0.9% saline solution was prepared and passed four times through a 25 gauge needle. Mice were killed by cervical dislocation and perfused through the left ventricle with 10 mls of iron oxide suspension. The kidneys were removed and sieved through a 75 μm sieve. The resulting suspension of glomeruli and tubules was washed three times with phosphate buffered saline (PBS) with centrifugation at 300 g and resuspended in 1 ml of DEPC treated PBS. Glomeruli were concentrated by washing three times with DEPC PBS under a magnetic field from a Dyna magnetic particle concentrator (MPC-E, Dynal, UK). Glomerular preparations obtained using this method routinely had a purity of $> 95\%$.

Reverse transcription polymerase chain reaction

Glomerular pellets from individual animals were snap frozen and stored at -70°C before mRNA extraction which was carried out with RNAzol B (Biogenesis, Poole, UK) following the manufacturer's instructions. The entire RNA sample from each animal was reverse transcribed with Moloney murine leukemia virus enzyme (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. The resulting cDNA was amplified by PCR using primers against NOS2 (735 bp product) and β-actin (202 bp) as reported previously [6] and for interleukin-1B (IL-1β; sense, 5′-GCA-ACT-GTC-CCT-GAA-CTC-A-3′; antisense, 5′-GCC-CAA-GGC-CAC-AGG-GAT-T-3′, 528 bp) and TNFα (sense, 5′-GGA-TCA-TCT-TCT-CAA-AAC-TCG-5′; antisense, 5′-TCA-CAG-AGC-AAT-GAC-TCC-AAA-3′, 468 bp). PCR was carried out for 35 (NOS2, β-actin) or 40 cycles with the following sequential steps: 72°C for one minute, 60°C for 30 seconds and 94°C for 30 seconds. The identity of the NOS2 PCR product was confirmed by restriction enzyme digestion with *Ava*I and with *Stu*I, which produced products of the predicted sizes. PCR products were analyzed on 1% agarose gel and visualized with ethidium bromide staining and ultraviolet light transillumination.

Histology

Kidneys were fixed in 10% buffered formal saline, paraffin embedded and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). For neutrophil counts sections were stained with chloroacetate esterase and a minimum of twenty glomeruli per section counted; results are expressed as neutrophils per glomerular cross section.

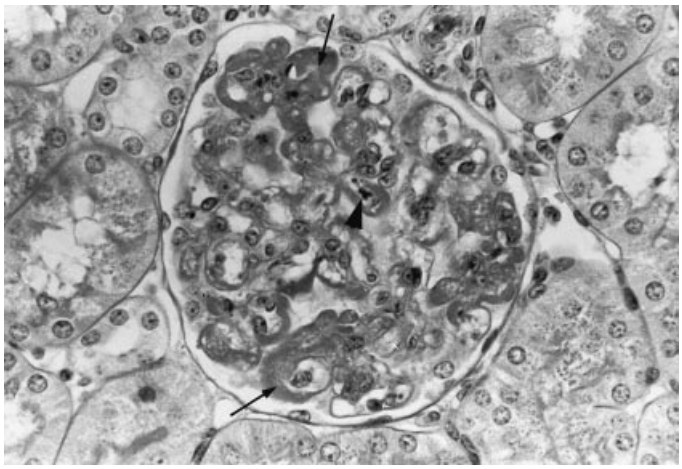

Fig. 1. Glomerulus 24 hours after induction of glomerulonephritis. There is prominent thrombosis (arrows). An infiltrating neutrophil is present (arrowhead) (periodic acid-Schiff ×500).

Glomeruli thrombi were scored as 0 to 4 for each glomerulus depending on the number of quadrants involved and a mean obtained for at least twenty glomeruli as previously described [16]

Immunohistochemistry

Kidney was fixed in paraformaldehyde, lysine, periodate fixative [23] for four hours at 4°C, washed in 7% sucrose overnight and frozen in isopentane cooled in liquid nitrogen. Tissue sections were cut and stained with monoclonal anti-mouse CD68 (FA/11; a kind gift from Dr. Andrew McKnight, Oxford, United Kingdom), to detect macrophages. The primary antibody was detected using biotinylated rabbit anti-rat antibody and streptavidin biotin peroxidase.

Statistics

Results are expressed as mean ± standard error and comparisons between groups are by Student's *t*-test.

RESULTS

Induction of glomerulonephritis

Immunization followed by anti-GBM antibody in the 129Sv wild type strain resulted in an accelerated nephrotoxic nephritis with persistent albuminuria up to 55 mg per 24 hours (normal 129Sv mice, albuminuria < 0.05 mg/24 hr). Histologically, at 24 hours there was neutrophil polymorphonuclear leukocyte infiltration and segmental glomerular thrombosis (Fig. 1). NOS2 mRNA was detected in glomeruli at 24 hours and nine days; NOS2 message was not present in normal glomeruli. From 6 to 9 days there was glomerular thrombosis and mild glomerular hypercellularity together with small cellular crescents in 10% of glomeruli (Fig. 2). There was focal tubular dilatation with eosinophilic casts and a focal, mild, interstitial mononuclear cell infiltrate. These changes increased in severity with increasing doses of anti-GBM antibody, and with 3 mg of antibody there was total glomerular thrombosis with non-perfusable kidneys.

The presence of neutrophils and a high level of albuminuria at

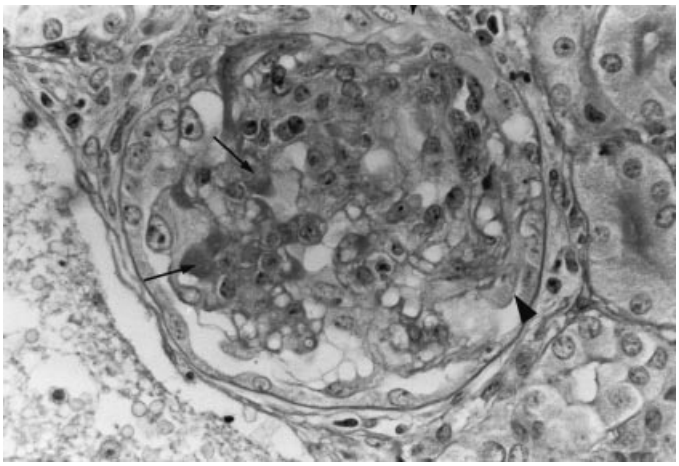

Fig. 2. Glomerulus six days after induction of glomerulonephritis. The glomerulus is hypercellular with thrombosis (arrows) and a developing crescent (arrowheads) (periodic acid-Schiff ×500).

24 hours suggested an early neutrophil-dependent phase of disease, preceding autologous disease. In experiments to assess the effect of NOS2 deficiency in these two phases, we therefore examined early (24 hr) injury, using a dose of 0.6 mg anti-GBM antibody, and autologous phase injury at six days using a lower dose of 0.3 mg. The lower dose was chosen to ensure significant survival at six days.

Experiment 1. Early injury (24 hours)

Disease was induced in 10 NOS2 mutant and 10 NOS2 heterozygous mice by immunization followed by 0.6 mg anti-GBM antibody. Two heterozygous animals died within 24 hours. At 24 hours mice were killed and three mice from each group were perfused with iron oxide to obtain isolated glomeruli for mRNA preparation and RT-PCR. Kidneys were taken for histology from all mice.

There were no significant differences in the histological appearances or level of albuminuria between the mutant and the heterozygous mice (Table 1) mice. NOS2 mRNA was induced in the glomeruli of heterozygous mice but was absent in NOS2 deficient mice (Fig. 3). IL-1β and TNF-α mRNA was induced in both strains of mice (Fig. 4). Disease in the NOS2 heterozygous mice did not differ significantly from that in wild type 129Sv mice.

Experiment 2. Autologous phase injury

Disease was induced in 12 NOS2 deficient and 12 NOS2 heterozygous mice by immunization followed by 0.3 mg anti-GBM antibody. One mutant mouse and 2 heterozygous mice died within 24 hours. Three mice from each group were killed at 24 hours for histological examination of kidneys. Two more mutant mice died on day five. The remaining mice were killed on day 6 and kidneys taken for histology and immunohistochemistry.

At 24 hours there was no difference in albuminuria or histological injury between the two groups (Table 1) as found in experiment 1. The autologous phase results are shown in Table 1. There was no significant difference in survival, level of albuminuria, or histological appearances between the groups by ranking for histological severity, scoring thrombi, crescent formation or macrophage infiltration.

DISCUSSION

Using a well characterized rabbit anti-mouse glomerular basement membrane antibody previously shown to induce heterologous disease [20, 24] we developed a model of accelerated anti-GBM nephritis in the 129Sv mouse. Accelerated anti-GBM glomerulonephritis depends on an autologous response to heterologous immunoglobulin, with anti-GBM specificity, planted on glomerular capillary walls. The accelerated model was chosen as injury in this model in the rat is known to be macrophage dependent [25]. The disease produced was similar to that in the rat with NOS2 induction, heavy albuminuria and a diffuse acute glomerulonephritis. The level of glomerular thrombosis and crescent formation was slightly higher than normally seen in the Lewis rat in our laboratory and the degree of macrophage infiltration was less. There have been several recent reports of this model in other strains of mice, stimulated by the availability of transgenic animals. It has been reported that the mechanisms of injury vary with strain; in C57BL/6 mice, cell mediated responses appear more important than antigen/antibody complexes [26], in contrast to BALB/c [26] and CSF-1 deficient (op/op) mice [27] where cell mediated mechanisms were not essential. As accelerated anti-GBM nephritis has not previously been reported in the 129Sv strain, which of these mechanisms of injury is most critical is unknown. The appearances in the glomeruli have features more in common with those described in the BALB/c strain with prominent thrombi and focal crescent formation but only a minor degree of macrophage infiltration.

In NOS2 deficient mice there was no induction of NOS2 message in glomeruli, contrasting with induction of NOS2 mRNA in the heterozygous and wild type mice during glomerulonephritis. Disruption of the NOS2 gene did not affect the level of injury in glomerulonephritis either at 24 hours or in the advanced autologous phase at six days. This lack of effect has also been found in a model of chronic glomerulonephritis: in a recent study on MRL-*lpr*/*lpr* mice, which develop a spontaneous lupus-like glomerulonephritis, targeted disruption of the NOS2 gene had no effect on glomerular disease although it protected against renal vasculitic lesions [28].

The role of NOS2 in several other inflammatory reactions has now been studied using NOS2 deficient mice. A clear and major role in the control of the intracellular parasites *Mycobacterium tuberculosis* [29], *Leishmania major* [19] and *Listeria monocytogenes* [30] has been demonstrated. However, the effects on other aspects of the inflammatory response are less clear. Acute dermal inflammation induced by carageenin was suppressed [19] while acute, acetic acid-induced colitis was enhanced [31] in mice lacking NOS2. In glomeruli we found no effect on neutrophil numbers. The macrophage influx in our model was also not affected by the lack of NOS2. This result is similar to that of MacMicking et al [29], who found no effect on granuloma formation in NOS2-deficient mice despite greatly reduced survival and increased bacterial load, and no impairment in the recruitment of macrophages in response to intraperitoneal thioglycollate. As in this previous report [29] there was no impairment of IL-1β and TNF-α induction.

Lack of NOS2 did not affect the prominent glomerular thrombosis in this model. NO inhibition with L-NAME in the rat has previously been associated with enhanced glomerular thrombosis

Table 1. Anti-GBM glomerulonephritis in NOS2-deficient and heterozygous mice

NOS2 phenotype	*N*	Cell infiltrate	Glomerular thrombi	Glomeruli with crescents %	Albuminuria mg/24 hours	Urine albumin/ creatinine ratio
Expt 1 (*24 hr*)		Neutrophils/glom				
Mutant	10	0.67 ± 0.14	2.1 ± 0.4	0	21 ± 7	6 ± 1
Heterozygous	8	0.59 ± 0.07	2.9 ± 0.2	0	18 ± 5	8 ± 2
Expt 2 (*24 hr*)						
Mutant	3	0.9 ± 0.2	1.7 ± 0.6	0	28 ± 4 ($N = 11$)	Not done
Heterozygous	3	0.6 ± 0.2	2.2 ± 0.8	0	21 ± 6 ($N = 10$)	Not done
Expt 2 (6 days)		Macrophages/glom				
Mutant	6	1.5 ± 0.3	2.0 ± 0.5	14 ± 5	12 ± 5	20 ± 2
Heterozygous	7	1.3 ± 0.2	1.7 ± 0.2	10 ± 4	18 ± 6	22 ± 4

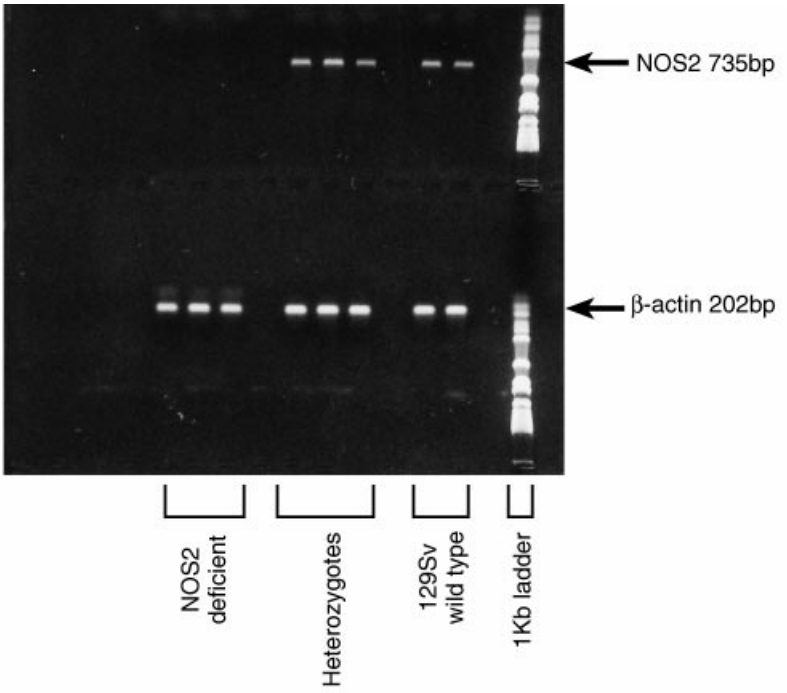

Fig. 3. Glomerular expression of NOS2 and β-actin mRNA, demonstrated by RT-PCR, 24 hours after induction of anti-GBM glomerulonephritis in NOS2-deficient, heterozygous and wild-type 129Sv mice.

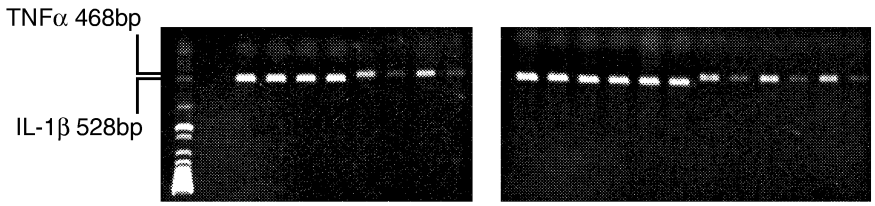

Fig. 4. Glomerular expression of interleukin 1B (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA demonstrated by RT-PCR 24 hours after induction of glomerulonephritis. The left hand panel shows heterozygous mice ($N = 2$) and the right hand panel NOS2 deficient mice ($N = 3$). For each mouse, and for each cytokine, two dilutions (1:5 and 1:15) of cDNA were used for PCR.

in endotoxin-induced injury [11, 32], and plasma arginine depletion using arginase increases glomerular thrombosis in acute nephrotoxic nephritis [16]. It has recently been reported that selective NOS2 inhibition with L-NIL in endotoxemia does not increase thrombi [33], which suggests a critical role for NOS3 rather than NOS2. It is currently unclear how much NO production can result locally from stimulation of endothelial NOS. Nitrite production is not detectable by Griess reaction (a relatively insensitive measure of NO production) in normal glomeruli [1], but it is known that this isoform is present in glomeruli [34, 35] and an increase in endothelial NOS mRNA has been reported in anti-Thy 1 glomerulonephritis [5]. It is important to emphasize that the role of NO in pathophysiology can only be clarified by distinguishing between the protective effects of constitutively generated NO and the potentially injurious effects of high levels of NO produced by NOS2

In conclusion, we have induced a model of autologous immune-mediated glomerulonephritis in mice, characterized by proteinuria and glomerular thrombosis with a minor degree of macrophage infiltration and focal crescent formation. There was glomerular induction of NOS2 mRNA. In NOS2-deficient mice there was no induction of NOS2 mRNA, but the disease did not

differ from that in mice with an intact NOS2 gene. Specifically we found an equivalent degree of albuminuria, glomerular thrombosis, neutrophil and macrophage accumulation, and crescent formation. Although we have not yet examined compensatory changes in other NOS isoforms, (NOS1 or NOS3) we can conclude from our current experiments that NOS2 does not play an essential role in the initiation or progression of these events in this form of glomerulonephritis in the mouse.

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